IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: WONG et al

Serial No. 09/856,339

Filed: 18 May 2001

For: PROCESS FOR OXIDISING TERPENES

DECLARATION

I, Dr Luet Wong, do hereby declare and state as follows:

1. I am a British subject of the Department of Chemistry, Inorganic Chemistry Laboratory, South Parks Road, Oxford OX1 3QR, UK. I presently have the position of University Lecturer in Chemistry. I have been working in the field of enzyme mutagenesis since 1990. My publications in this field are shown in annex I.

2. I am one of the inventors named for US Patent Application No. 09/856,339. I understand that the US Patent Office Examiner has objected that the oxidation method described in the claims of this patent application is obvious from the disclosure of GB-A-2294692, GB-A-2306485, US-A-6117661 and US-A-6100074 (hereafter referred to as the "cited documents"). I have been asked to comment on the Examiner's objection.

3. The claims of the application which are currently under consideration concern oxidation by particular mutant monooxygenase enzymes of limonene, pinene or a cyclic sesquiterpene; or a substituted derivative thereof, with the proviso that the substituent is not a halogen or does not comprise an oxygen atom. When mutant P450cam enzymes are used they are required to have particular combinations of mutations.

4. The oxidation of limonene, pinene or cyclic sesquiterpenes by mutant P450cam monoxygenases with particular combinations of mutations is not discussed in the cited documents. Table 6 on page 47 of the application shows the oxidation of limonene, pinene and a sesquiterpene by enzymes with particular combinations of mutations. Bell et al (2003) J. Am. Chem. Soc. 125, 705-14 investigates the oxidation of pinene by P450cam enzymes with different combinations of mutations. As can be seen from the third and fourth row of figures in table 2 on page 709 of this document enzymes with the particular combination of multiple mutations specified in the present claims have a higher production rate and a higher coupling efficiency (compare the figures for Y96F versus the figures to the right). As can be seen from page 41 of the present application pinene and limonene have very similar structures and therefore this data would also be expected to apply to limonene.

5. Table 6 of the application also shows how the use of enzymes with different specific combinations of mutations can be used to alter the oxidation products which are produced in the reaction. This is true for limonene, pinene and the sesquiterpene valencene. The sesquiterpene work described in Table 6 has now been published in Sowden et al (2005) Org.

Biomol. Chem 3, 57-64. This publication discusses the increased rates of reactions (see for example first full paragraph left hand column page 59) as well as the fact that different combinations of mutations give different oxidation products (see first two full paragraphs right hand column page 59 and table 1 on page 58). The cited documents do not provide any data showing oxidation with enzymes comprising the specific combinations of mutations specified in the present claims or any suggestion that enzymes with different combinations of mutations generate different oxidation products.

6. The Examiner refers to the fact that the cited documents mention isoprene and monoterpenes, and that these molecules are structurally related to sesquiterpenes. Whilst the sesquiterpenes are structurally related to isoprene and monoterpenes, this does not mean that an enzyme which oxidised isoprene or a monoterpene would be expected to oxidise a sesquiterpene. A sesquiterpene is a considerably larger molecule than isoprene or a monoterpene. The claims now refer only to cyclic sesquiterpenes. The presence of a ring within a molecule structure will cause the molecule to be much less flexible and to adopt a rigid three dimensional structure. This lack of flexibility often leads to a molecule becoming more resistant to chemical change as it has a decreased ability to change conformation during a reaction.

7. However more importantly in the present case the lack of flexibility in the substrate molecule means that the active site of the P450cam enzyme needs to be flexible enough for the relevant amino acids to contact the substrate and take part in the oxidation reaction. Figure 3 of Bell et al shows the manner in which the active site of P450cam surrounds the substrate molecule. Line 7 right hand column of page 709 of this document also notes that it was found that the monoterpene pinene was "not freely mobile within the active site". Thus the active site of P450cam closely associates with a monoterpene substrate.

8. Thus even if the cited documents were interpreted as disclosing oxidation of isoprene or a monoterpene by P450cam enzyme, it would not be expected that a cyclic sesquiterpene could be oxidised by this enzyme. It would not have been expected that a molecule with the high three dimensional volume of a cyclic sesquiterpene could fit into an active site which is designed to contain much smaller molecules (an active site in which the monoterpene pinene is not mobile), and that a molecule which is as rigid as a cyclic sesquiterpene could be oxidised by efficiently by a P450cam enzyme. The large aromatic compounds disclosed in the cited documents would have a flat planar structure, and thus could not be used to predict whether or not a sesquiterpene could be oxidised by a P450cam enzyme. Flat planar structures can more easily fit into a given space than a rigid three dimensional structure.

9. Thus the knowledge that isoprene and a monoterpene can be oxidised by P450cam cannot be used to predict that a sesquiterpene could also be oxidised by P450cam. These molecules are similar at a chemical level, but have very different structural and physical properties which would be expected to cause differences in susceptibility to oxidation by P450cam. Whilst the cited documents discuss the desirability of introducing less polar amino acids into the active site of P450cam or even amino acids of different size, they do not give any indication that mutations in P450cam may be used to allow a molecule the size of a sesquiterpene to enter the active site, or that such mutant enzymes have the flexibility to oxidise a cyclic sesquiterpene. Such concepts are not discussed in the cited documents, and thus these documents give no reason to expect that a sesquiterpene will be successfully oxidised by a mutant P450cam enzyme.

10. Mutants of P450BM-3 are not disclosed in the cited documents. Annexes II, III and IV show that the mutant P450BM-3 enzymes mentioned in the claims have a high activity towards limonene, pinene and a sesquiterpene (valencene).

11. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of this declaration, the patent application, or any patents issuing thereon.

Signed Luet Wong
Dr Luet Wong

This 13 Day of May 2005.

ANNEX I

PUBLICATIONS

(1) "η-Benzenebis(trimethylphosphine)iron as a precursor to $Fe(\eta\text{-}C_5H_5)(PMe_3)_2$ derivatives, R = H, Me: the equilibrium $[Fe](PMe_3)Et = [Fe](\eta\text{-}C_2H_4)H + PMe_3$ where $[Fe] = Fe(\eta\text{-}C_5H_5)(PMe_3)$."

M.L.H. Green and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1984**, 1442-1443.

(2) "η-Benzenebis(trimethylphosphine)iron as a precursor to $Fe(\eta\text{-}C_5H_5)(PMe_3)_2$ derivatives, R = H, Me: the equilibrium $[Fe](PMe_3)Et = [Fe](\eta\text{-}C_2H_4)H + PMe_3$ where $[Fe] = Fe(\eta\text{-}C_5H_5)(PMe_3)$."

M.L.H. Green and L.-L. Wong
J. Chem. Soc., Dalton Trans., **1987**, 411-416.

(3) "η-Benzenebis(trimethylphosphine)iron: A useful precursor to polyene iron derivatives. Crystal structure of 2-4-η:2'-4'-η-Bi(cyclohex-3-en-2-yl)-bis(trimethylphosphine)iron(II)."

M.L.H. Green, D. O'Hare, and L.-L. Wong
J. Chem. Soc., Dalton Trans., **1987**, 2031-2038.

(4) "The reaction of $[W(PMe_3)_4(\eta^2\text{-}CH_2PMe_2)H]$ with CO_2 and H_2: Characterisation of $[\{W(PMe_3)_3(\eta^1\text{-}PMe_2CH_2\}_2(C_3H_2O_6)]$ using two-dimensional nuclear magnetic resonance Spectroscopy."

A.E. Derome, M.L.H. Green, G. Parkin, D. O'Hare, and L.-L. Wong
J. Organomet. Chem., **1986**, *317*, 61-68.

(5) "Carbon-hydrogen-transition metal bonds."

M. Brookhart, M.L.H. Green, and L.-L. Wong
Prog. Inorg. Chem., **1988**, 36, 1-124.

(6) "Is a 16e η^1-ethyl intermediate necessary for hydrogen scrambling in ethylene-hydride complexes?"

M.L.H. Green and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1988**, 677-679.

(7) "Formation of three-vertex metalloboranes from mono-borane precursors: X-Ray crystal structures of $[Mo(\eta\text{-}C_5H_5)_2H(\eta^2\text{-}B_2H_5)]$ and $[Ru(\eta\text{-}C_5Me_5)(PMe_3)(\eta^2\text{-}B_2H_7)]$."

P.D. Grebenik, M.L.H.Green, M.A, Kelland, J.B. Leach, P. Mountford, G. Stringer, N.M. Walker, and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1988**, 799-801.

(8) "η^3- and η^7-Cycloheptatrienyltungsten Chemistry: Synthesis of $[W(\eta^5\text{-}C_7H_9)(\eta^3\text{-}C_7H_7)(PMe_3)_2]$ and $[W(\eta\text{-}C_7H_7)(\eta^3\text{-}C_7H_{11})(PMe_3)]$."

M.L.H. Green, D.K. Siriwardene, D. O'Hare, and L.-L. Wong
J. Chem. Soc., Dalton Trans., **1988**, 2851-2854.

(9) "Is a 16e η^1-ethyl intermediate necessary for hydrogen scrambling in ethylene-hydride complexes?"

M.L.H. Green and L.-L. Wong
Nouv. J. Chem., **1989**, *13*, 747-753.

(10) "Hydrogen scrambling processes in [Ta(η-C_5Me_5)(η-C_5H_5){μ-H)$_2BH_2$}]: A surprising observation."

M.L.H. Green and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1989**, 571-573.

(11) "A new mechanism for exchange processes observed in the compounds [M(η-C_5H_5)$_2$(*exo*-η-RCH=CH_2)H], M = Nb and Ta."

J.E. Bercaw, B.J. Burger, M.L.H. Green, B.D. Santasiero, A. Sella, M.S. Trimmer, and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1989**, 734-736.

(12) "Substitution reactions of the zerovalent iron complexes [Fe(η-C_6H_6)(PMe_3)$_2$] and [Fe(η-C_6H_6)(dmpe)] (dmpe = $Me_2CH_2CH_2PMe_2$)."

L. Howarth and L.-L.Wong
J. Chem. Soc., Dalton Trans., **1989**, 1385-1391.

(13) "Hydrogen scrambling processes in [Ta(η-C_5Me_5)(η-C_5H_5){μ-H)$_2BH_2$}]: A surprising observation."

M.L.H. Green and L.-L. Wong
J. Chem. Soc., Dalton Trans., **1989**, 2133-2138.

(14) "Some chemistry of half-sandwich η-arene tungsten compounds."

M.L.H. Green, A.K. Hughes, P. Lincoln, J.J. Martin-Polo, P. Mountford, A. Sella, L.-L. Wong, J.A. Bandy, T.W. Banks, K. Prout, and D.J. Watkin
J. Chem. Soc., Dalton Trans., **1992**, 2063-2069.

(15) "Mechanism for the exchange processes observed in the compounds [M(η-C_5H_5)$_2$(*exo*-η-RCH=CH_2)H], M = Nb and Ta."

M.L.H. Green, A. Sella, and L.-L. Wong
Organometallics, **1992**, *11*, 2650-2659.

(16) "The relationship between intramolecular chemical exchange and NMR-observed rate constants."

M.L.H. Green, L.-L. Wong, and A. Sella
Organometallics, **1992**, *11*, 2660-2668.

(17) "Nuclear magnetic resonance studies on partially deuteriated transition metal-methyl derivatives"

M.L.H. Green, A.K. Hughes, N.A. Popham, A.H.H. Stephens, and L.-L. Wong
J. Chem. Soc., Dalton Trans., **1992**, 3077-3082.

(18) "Synthesis, structure and dynamic behaviour of the compound [Nb(η-C_5H_5)$_2$(σ-C_5H_5)(NBu^t)]"

M.L.H. Green, D.M. Michaelidou, P. Mountford, A.G. Suárez, and L.-L. Wong
J. Chem. Soc., Dalton Trans., **1993**, 1593-1597.

(19) "Electron transfer in ruthenium/zinc porphyrin derivatives of recombinant human myoglobins. Analysis of tunneling pathways in myoglobin and cytochrome *c*."

D.R. Casimiro, L.-L. Wong, J.L.Colón, T.E. Zewert, I.-J. Chang, J.R. Winkler, and H.B. Gray
J. Am. Chem. Soc., **1992**, *115*, 1485-1489.

(20) "Metal Ions in Biological Fluids"

P. de Oliveira, H.A.O. Hill and L.-L. Wong
Handbook on metal-ligand interactions in biological fluids, Ed. G. Berthon, Marcel Dekker, **1994**.

(21) "Cytochrome $P450_{cam}$ monooxygenase can be redesigned to catalyse the regioselective aromatic hydroxylation of diphenylmethane"

S.M. Fowler, P.A. England, A.C.G. Westlake, D.A. Rouch, D.P. Nickerson, C. Blunt, D. Braybrook, S. West, L.-L. Wong, and S.L. Flitsch
J. Chem. Soc., Chem. Commun., **1994**, 2761-2762.

(22) "The catalytic activity of human myoglobin is enhanced by a single active-site mutation: F43Y"

D.C. Levinger, J.-A. Stevenson, and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1995**, 2305-2306.

(23) "Aliphatic *vs.* aromatic C-H bond activation of phenylcyclohexane catalysed by cytochrome $P450_{cam}$"

P.A. England, D.A. Rouch, A.C.G. Westlake, S.G. Bell, D.P. Nickerson, M. Webberley, S.L. Flitsch, and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1996**, 357-358.

(24) "*N*-(2-Ferrocene-ethyl)maleimide: A new electroactive sulphydryl-specific reagent for cysteine-containing peptides and proteins"

K. Di Gleria, H.A.O. Hill, and L.-L. Wong
FEBS Lett., **1996**, *390*, 142-144.

(25) "Direct electrochemistry of cytochrome $P450_{cam}$"

J. Kazlauskaite, A.C.G. Westlake, L.-L. Wong, and H.A.O. Hill
J. Chem. Soc., Chem. Commun., **1996**, 2189.

(26) "Engineering the selectivity of aliphatic C-H Bond oxidation catalysed by cytochrome $P450_{cam}$"

N.E. Jones, P.A. England, D.A. Rouch, and L.-L. Wong
J. Chem. Soc., Chem. Commun., **1996**, 2413.

(27) "The catalytic oxidation of linear and branched alkanes by cytochrome $P450_{cam}$"

J.-A. Stevenson, A.C.G. Westlake, C. Whittock, and L.-L. Wong
J. Am. Chem. Soc., **1996**, *118*, 12846-12846.

(28) "The catalytic activity of cytochrome $P450_{cam}$ towards styrene oxidation is increased by site specific mutagenesis"

D.P. Nickerson, C.F. Harford-Cross, S.R. Fulcher, and L.-L. Wong
FEBS Lett., **1997**, *405*, 153-156.

(29) "Protein engineering of cytochrome $P450_{cam}$"

L.-L. Wong, A.C.G. Westlake and D.P. Nickerson
Structure and Bonding, **1997**, *88*, 175-207.

(30) "Selective aliphatic and aromatic carbon-hydrogen bond activation catalysed by mutants of $P450_{cam}$".

S.G. Bell, D.A. Rouch, and L.-L. Wong
J. Mol. Catal. B: Enzymol., **1997**, *3*, 285-292.

(31) "The dimerisation of *Pseudomonas putida* cytochrome $P450_{cam}$: practical consequences and engineering of a monomeric enzyme,"

D.P. Nickerson and L.-L. Wong
Protein Eng., **1997**, *10*, 1357-1361.

(32) "An improved procedure for the preparation of X-ray diffraction quality crystals of cytochrome $P450_{cam}$."

D.P. Nickerson and L.-L. Wong
Acta Cryst. **1997**, *D54*, 470-472.

(33) "Covalent attachment of an electroactive sulphydryl reagent in the active site of cytochrome $P450_{cam}$ as revealed by the crystal structure of the modified protein."

K. Di Gleria, D.P. Nickerson, H.A.O. Hill, L.-L. Wong, and V. Fulop
J. Am. Chem. Soc., **1998**, *120*, 46-52.

(34) "Cytochrome P450 Monooxygenases"

L.-L. Wong
Curr. Opin. Chem. Biol., **1998**, *2*, 263-268 .

(35) "The oxidation of naphthalene and pyrene by cytochrome $P450_{cam}$."

P.A. England, C.F. Harford -Cross, J.-A. Stevenson, D.A. Rouch, and L.-L. Wong
FEBS Lett., **1998**, *424*, 271-274.

(36) "Engineering molecular recognition in alkane oxidation catalysed by cytochrome $P450_{cam}$."

J.-A. Stevenson, J.K. Bearpark and L.-L. Wong
New J. Chem., **1998**, 551-552.

(37) "Surface-modified mutants of cytochrome $P450_{cam}$: enzymatic properties and electrochemistry"

K.K.-W. Lo, L.-L. Wong and H.A.O. Hill
FEBS Lett., **1999**, *451*, 342-346.

(38) "The thermodynamics and kinetics of electron transfer in the cytochrome $P450_{cam}$ enzyme system"

M.J. Honeychurch, H.A.O. Hill, and L.-L. Wong
FEBS Lett., **1999**, *451*, 351-353.

(39) "Mutation of Glu-84 at the putative potassium binding site affect camphor binding and oxidation by cytochrome $P450_{cam}$"

A.C.G. Westlake, C.F. Harford-Cross, J. Donovan and L.-L. Wong
Eur. J. Biochem., **1999**, *265*, 929-935.

(40) "Protein engineering of cytochrome $P450_{cam}$ for the oxidation of polycyclic aromatic hydrocarbons"

C.F. Harford-Cross, P.A. England, F.K. Allan, A.B. Carmichael, D.A. Rouch, and L.-L. Wong
Prot. Engng., **2000**, *13*, 121-128.

(41) "The oxidation of polychlorinated benzenes by genetically engineered cytochrome $P450_{cam}$: potential applications in bioremediation"

J.P. Jones, E.J. O'Hare and L.-L. Wong
Chem. Comm., **2000**, 247-248.

(42) "Mutations of Phe-193 in the putative substrate access channel of cytochrome $P450_{cam}$ dramatically alters the substrate specificity"

J.-A. Stevenson, J.P. Jones, and L.-L. Wong
Israel J. Chem., **2000**, *40*, 55-62.

(43) "Catalytic reductive dehalogenation of hexachloroethane by molecular variants of cytochrome $P450_{cam}$ (CYP101)"

M.E. Walsh, P. Kyritsis, N.A.J. Eady, H.A.O. Hill, and L.-L. Wong
Eur. J. Biochem., **2000**, *267*, 5815-5820.

(44) "A scanning tunnelling study of immobilised cytochrome $P450_{cam}$"

J.J. Davis, D. Djuricic, K.K.W. Lo, E.N.K. Wallace, L.-L. Wong and H.A.O. Hill
Faraday Discuss., **2000**, *116*, 15-22.

(45) "Oxidation of polychlorinated benzenes by genetically engineered CYP101 (cytochrome $P450_{cam}$)"

J.P. Jones, E.J. O'Hare, and L.-L. Wong
Eur. J. Biochem., **2001**, *268*, 1460-1467.

(46) "Engineering the haem monooxygenase cytochrome $P450_{cam}$ for monoterpene oxidation"

S.G. Bell, R.J. Sowden, and L.-L. Wong
Chem. Commun., **2001**, 635-636.

(47) "Protein engineering of *Bacillus megaterium* CYP102 : The oxidation of polycyclic aromatic hydrocarbons"

A.B. Carmichael and L.-L. Wong
Eur. J. Biochem., **2001**, *268*, 3117-3125.

(48) "Direct electrochemistry of pentachlorophenol hydroxylase"

W. Xie, J.P. Jones, L.-L. Wong and H.A.O. Hill
Chem. Commun., **2001**, 2370-2371.

(49) "Engineering the CYP101 system for *in vivo* oxidation of unnatural substrates"

S.G. Bell, Charles Harford-Cross, and L.-L. Wong
Prot. Engng., **2001**, *14*, 797-802.

(50) "Butane and propane oxidation by engineered cytochrome $P450_{cam}$"

S.G. Bell, J.-A. Stevenson, H.D. Boyd, S. Campbell, A.D. Riddle, E.L. Orton, and L.-L. Wong
Chem. Commun. **2002**, 490-491.

(51) "A scanning tunnelling microscopy (STM) investigation of complex formation between cytochrome $P450_{cam}$ and putidaredoxin"

D. Djuricic, H.A.O. Hill, K. K.-W. Lo, and L.-L. Wong
J. Inorg. Biochem., **2002**, 88, 362-367.

(52) "A molecular level study of complex formation between putidaredoxin and cytochrome P450 by scanning tunnelling microscopy"

R. Mukhopadhyay, L.-L. Wong, K.K.-W. Lo, T. Pochapsky, and H.A.O. Hill
PCCP, **2002**, *4*, 641-646.

(53) "The electrochemistry and scanning tunnelling microscopy of the flavoprotein, putidaredoxin reductase, from *Pseudomonas putida*"

W.M McGee, D. Djuricic, K. Lorimer, L.-L. Wong, and H.A.O. Hill
J. Electroanal. Chem., **2002**, *538-539*, 261-265.

(54) "The crystal structure of the F87W/Y96F/V247L mutant of cytochrome $P450_{cam}$ with 1,3,5-trichlorobenzene bound and further protein engineering for the oxidation of pentachlorobenzene and hexachlorobenzene"

X. Chen, A. Christopher, J.P. Jones, S.G. Bell, Q. Guo, F. Xu, Z. Rao, and L.-L. Wong
J. Biol. Chem., **2002**, *277*, 37519-37526.

(55) "Molecular recognition in (+)-α-pinene oxidation by cytochrome $P450_{cam}$"

S.G. Bell, X. Chen. R.J. Sowden, F. Xu, J.N. Williams, L.-L. Wong, and Z. Rao
J. Am. Chem. Soc., **2003**, *125*, 705-714.

(56) "Engineering cytochrome $P450_{cam}$ into an alkane hydroxylase"

S.G. Bell, Erica L. Orton, H. Boyd, J.-A. Stevenson, A. Riddle, S. Campbell, and L.-L. Wong
Dalton Trans., **2003**, 2133-2140.

(57) "The electrochemistry and scanning tunnelling microscopy of the flavoprotein putidaredoxin reductase on alkanethiol-modified gold."

E.A. Bentley, Y. Astier, W. Ji , S.G. Bell, L.-L. Wong and H. A. O. Hill
Inorg. Chim. Acta, **2003**, *356*, 343-348.

(58) "Engineering substrate recognition in catalysis by cytochrome P450cam."

S.G. Bell, X. Chen, F. Xu, Z. Rao and L.-L. Wong,
Biochem. Soc. Trans., **2003**, 31, 558-562.

(59) "Redox properties of cytochrome $P450_{BM3}$ measured by direct methods."

B.D. Fleming, Y. Tian, S.G. Bell, L.-L. Wong, V. Urlacher and H.A.O. Hill
Eur. J. Biochem., **2003**, *270*, 4082-4088.

(60) "Orientated immobilisation of Pseudomonas putida putidaredoxin on a gold(111)-buffer interface: A real time scanning tunnelling microscopy study."

R. Mukhopadhyay, , K.K.-W. Lo, L.-L. Wong, and H.A.O. Hill
J. Microscopy, **2004**, *213*, 6-10.

(61) "Crystal structure of human pirin: an iron-binding nuclear protein and transcription cofactor"

H. Pang, M. Bartlam, Q. Zeng, H. Miyatake, T. Hisano, K. Miki, L.-L. Wong, G.F. Gao, and Z. Rao
J. Biol. Chem., **2004**, *279*, 1491-1498.

(62) "Heme proteins: Mono- and di-oxygenases"

L.L. Wong and S.G. Bell
In "*Encyclopaedia of Inorganic Chemistry*", 2nd Edn., in press.

(63) "Novel mutants of cytochrome $P450_{cam}$ with high-spin hemes in the absence of substrate"

S.G. Bell, A. Insley, F. Xu, A. Daggers, Z. Rao, and L.-L. Wong, submitted.

Annex II

Activities for the oxidation of the enantiomers of limonene by $P450_{BM-3}$ mutants

$P450_{BM-3}$ Enzyme	**NADPH Consumption Rate** [a]	**Product Formation Rate** [a]	**Coupling Efficiency**
Wild-type	189 (77)	64 (29)	37% (37%)
R47L/Y51F	461 (386)	263 (168)	59% (44%)
R47L/Y51F/F87A	360 (307)	120 (105)	32% (34%)

The results are the average of identical (at least three) reactions whose results coincide and have a standard deviation of <5%.

The results for (–)-*S*-limonene are given in brackets below those for the (+)-*R*-isomer.

[a] Rates are given as nmol (nmol P450)$^{-1}$ min^{-1}.

[b] A spin-state shift to 5% (10%) hs was seen.

Annex III

Activities for the oxidation of (+)-α-pinene by $P450_{BM-3}$ mutants

$P450_{BM-3}$ Enzyme	**NADPH Consumption Rate [a]**	**Product Formation Rate [a]**	**Coupling Efficiency**
Wild-type	14	5	39%
R47L/Y51F/F87A	92	54	59%

The results are the average of identical (at least three) reactions whose results coincide and have a standard deviation of <5%.

[a] Rates are given as nmol (nmol P450)$^{-1}$ min^{-1}.

[b] A spin-state shift to 55% hs was seen.

ANNEX IV

Activities for the oxidation of (+)-valencene and (+)-nootkatone by the $P450_{BM-3}$ base mutants

$P450_{BM-3}$ Enzyme	**NADPH Consumption Rate** [a]	**Product Formation Rate** [a]	**Coupling Efficiency**
Wild-type	40 (516)	12 (112)	30% (21%)
F87A [b]	83 (218)	28 (75)	30% (34%)
R47L/Y51F	45 (296)	30 (79)	66% (27%)

The results are the average of identical (at least three) reactions whose results coincide and have a standard deviation of <5%.

The results for (+)-nootkatone are given in brackets beneath those for (+)-valencene.

[a] Rates are given as nmol(nmol P450)$^{-1}$min^{-1}.

[b] A spin-state shift to 10% (25%) hs was seen.

[c] A spin-state shift to (35%) hs was seen.